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A SEROLOGICAL SURVEY OF IOWA STOCK COWS FOR ANTIBODIES TO INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS

by

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INTRODUCTION

Any individual who undertakes a research project may be motivated by his curiosity to learn of the mystery in which the object of his search is bound and shrouded. Or, he may be driven to certain research to prove or disprove an hypothesis. The author, wishing to test an idea concerning the incidence of an infectious disease currently affecting cattle, undertook the research described here to study the stock cow population in Iowa to ascertain the frequency of an upper respiratory disease, infectious bovine rhinotracheitis.

Even though infectious bovine rhinotracheitis was not recognized as a distinct disease entity until 1955, it had existed as an acute upper respiratory infection of undetermined etiology in feedlot cattle and dairy cows of the western and midwestern parts of the United States for several years.

A major problem in ascertaining the frequency of infectious bovine rhinotracheitis is its resemblance to mucosal disease and virus diarrhea. Due to the similarities of these diseases, it appeared that they might be caused by a common agent or closely related agents. It has been determined that infectious bovine rhinotracheitis is caused by a distinctly different virus than that which causes mucosal disease or virus diarrhea, which are presently referred to as the mucosal disease complex.

At the beginning of this project, no one had made a

serological survey to determine the incidence of infectious bovine rhinotracheitis in cattle in the United States. A commercial laboratory had summarized the results of their diagnostic service. However, this was not a true representation of the incidence of infectious bovine rhinotracheitis, as most of the sera were submitted because the patient was suspected of harboring an infection. Knowledge of the incidence of infectious bovine rhinotracheitis did not exist. This was the second reason which motivated this study.

A unique opportunity to measure the incidence of infectious bovine rhinotracheitis in Iowa stock cow herds became available with the Cooperative State-Federal Brucellosis Testing Program. Thousands of blood serum samples drawn from herds in all parts of the state thus were available for additional testing. Numbers were sufficient to allow a statistically designed survey. The State-Federal Brucellosis Laboratory and the Iowa Veterinary Diagnostic Laboratory are housed in adjacent rooms in the same building. The stage was set for the research project described here--A Serological Survey of Iowa Stock Cows for Antibodies to Infectious Bovine Rhinotracheitis Virus.

REVIEW OF THE LITERATURE

The first account of infectious bovine rhinotracheitis as a disease entity was reported from Colorado by Miller (1955). Miller referred to the disease as "infectious necrotic rhinotracheitis." The disease had been under surveillance for a little more than 4 years in Colorado feedlot cattle. Miller's account calls attention to clinical and postmortem observations. The affected animals had elevated temperature, blood-tinged nasal discharge, salivation, dyspnea, conjunctivitis and lacrimation. The nasal mucosa was hyperemic. There was corneal opacity. A diphtheritic membrane was present in the trachea. Milk production was reduced in lactating females.

Schroeder and Moys (1954) noted an acute upper respiratory infection in California dairy cattle. They made no acknowledgement of the disease in Colorado. Perhaps the two diseases were too dissimilar to be recognized as one and the same. They did not observe ocular involvement. In a report at the United States Livestock Sanitary Association meeting, McKercher et al. (1954) summarized their findings concerning a new influenza-like disease in cattle. Quite likely they were observing the same disease as Schroeder and Moys had reported earlier.

Once the alarm had been sounded, reports of similar conditions occurring in cattle in other parts of United States and the world began to appear. McKercher (1955), representing his staff of investigators, addressed the next annual meeting

of the United States Livestock Sanitary Association at which time he recognized that the disease in California was very similar to that which Jensen et al. (1955) reported in Colorado.

Gillespie et al. (1957) conducted animal inoculation and virus isolation studies using infectious bovine rhinotracheitis virus strains which were isolated by researchers in the western United States. Gillespie also had evidence that the disease had been present in New Jersey as early as 1941. Blood which was collected from a calf on this date was tested in 1957 and found to have antibodies to infectious bovine rhinotracheitis virus.

McKercher and Straub (1960) made an isolation from Utah range cattle. Chow (1961) isolated the virus from calves from 2 herds of range cattle in Colorado.

Maré and van Rensburg (1961) repeatedly isolated a virus quite similar to infectious bovine rhinotracheitis virus from cases of infectious infertility (epivag) of South African cattle.

A virus was isolated by French (1962a) from Australian calves affected with an encephalitis. Shortly thereafter, French (1962b) established the relationship of this virus to infectious bovine rhinotracheitis virus by physical and serological characteristics.

Studdert et al. (1961) published an account of infectious bovine rhinotracheitis in Ontario, Canada. Chamberland et al. (1963) observed outbreaks of infectious bovine rhinotracheitis

in 4 herds of dairy cattle in Quebec.

Neutralizing antibody for infectious bovine rhinotracheitis virus was demonstrated in cattle in southern Japan by Yamada et al. (1964)

As reports of infectious bovine rhinotracheitis were made, it soon became apparent that the disease was spread statewide, nationwide and worldwide. The symptoms and lesions described were quite varied. Although these different conditions were called infectious bovine rhinotracheitis, some had little or no resemblance to an infectious tracheitis. However, the diagnosis was based on the isolation and identification of the virus.

Several papers were written by authors who observed only an upper respiratory infection. Others observed conjunctivitis without respiratory involvement. And still other workers observed abortion as the only manifestation of the disease. One will note the marked difference in characteristics of the disease in the references of French and Maré and van Rensburg.

The virus was isolated by Abinanti and Plumer (1961) from a case of conjunctivitis. Within a week two thirds of a herd of Aberdeen Angus feeder cattle were affected with conjunctivitis. They reproduced the disease with viral isolates from the herd and with known infectious bovine rhinotracheitis virus. Barenfus et al. (1963) described an outbreak of meningoencephalitis in calves in Los Angeles county, Calif., from which infectious bovine rhinotracheitis virus was isolated.

The lesions and behavior of the affected animals differed little from any other case of meningoencephalitis caused by other agents.

Van Kruiningen and Wolke (1963) observed an outbreak of infectious bovine rhinotracheitis in a dairy herd in New York. There was nasal and lacrimal exudation. Body temperature was elevated. Milk production diminished and ceased altogether in some cows.

Another outbreak in dairy cattle in New York was reported by Van Kruiningen and Bartholomew (1964). The usual symptoms, nasal and ocular exudation, elevated body temperature, salivation, dyspnea and cough, were observed. There was some loss in milk production and 2 abortions. One calf died. The lungs, as described by Van Kruiningen and Bartholomew, were congested, firmer than normal and red. The viscera were coated with a fibrinous exudate. The liver was mottled by white foci about 1 mm. in diameter. There were raised reddish-black hemorrhagic spots 1 to 2 mm. in diameter in the abomasal mucosa anterior to the pylorus. Grayish-white irregular clumps of caseous material adhered to the rumen mucosa. Intranuclear inclusions were found in the epithelial cells adjacent to necrotic foci in the rumen.

A severe case of keratoconjunctivitis was observed by Hughes et al. (1964) in California cattle. It resembled bovine infectious keratitis, but corneal ulceration was absent. There was corneal opacity and vascularization. The ocular exudation

was excessive and the hair on the face became matted. Infectious bovine rhinotracheitis virus was isolated from the ocular exudate.

The disease which was observed by Chow et al. (1955), Gillespie et al. (1957), Jensen et al. (1955), McIntyre (1954), Miller (1955), Schroeder and Moys (1954) and McKercher et al. (1954) was very characteristic of what the name implies--infectious bovine rhinotracheitis. Nasal exudate and hemorrhagiconecrotic tracheitis were noted to occur with the greatest frequency.

McKercher et al. (1955) thought that "rhinotracheitis", the term which was originally used by the Colorado group, would be very appropriate as a name for the disease and suggested that "infectious" and "bovine" be incorporated in the name so as to reflect its true characteristics. Hence, another disease, infectious bovine rhinotracheitis, was added to the steadily growing list of new bovine viral diseases.

Gillespie et al. (1959) unexpectedly found that infectious pustular vulvovaginitis virus (described by Kendrick et al., 1958) was neutralized by serum from a calf which had recovered from infectious bovine rhinotracheitis. How could these two viruses, infectious pustular vulvovaginitis and infectious bovine rhinotracheitis virus, which caused clinical diseases of such great dissimilarity have any common characteristics! A comparative study of the two viruses was promptly undertaken.

Calves which had been injected with an infective dose of infectious bovine rhinotracheitis virus and allowed to recover were resistant to a subsequent challenge dose of virus. Likewise, calves which had been injected with an infective dose of infectious pustular vulvovaginitis virus and allowed to recover were resistant to a subsequent challenge dose of that virus. Each virus immunized calves against itself and against each other, and each virus was neutralized by its own antiserum and antiserum of the other virus. This was as they had expected to find it.

Gillespie's group also noted that both of the viruses behaved almost identically when inoculated into cattle by the same route. Similar serum titers were achieved with both viruses. They concluded that the two viruses, infectious bovine rhinotracheitis and infectious pustular vulvovaginitis, were one and the same.

This discovery was baffling to Gillespie and his colleagues. They were quite familiar with infectious pustular vulvovaginitis as a clinical disease, but they had no first-hand knowledge of infectious bovine rhinotracheitis. It had never been diagnosed before in New York State. And yet, in 1957, serum from 12 percent of 43 herds of dairy cattle contained neutralizing antibodies to infectious bovine rhinotracheitis virus. Similarly, there were relatively few clinical cases of vaginitis observed; however, in 1958, serum from 15 percent of 53 herds of cattle had antibodies to infectious

pustular vulvovaginitis virus. It became even more confusing when the two viruses were neutralized by antibodies to the other. Before fully accepting this new concept, Gillespie was cautious and suggested that the interrelationship of infectious pustular vulvovaginitis be given further study.

Within a short time after the work of Gillespie and his collaborators, infectious bovine rhinotracheitis was observed in the eastern United States with greater frequency. Baker et al. (1960) thought that the virus was capable of causing disease other than upper respiratory infection. They studied the response of newborn calves to the virus. The calves became depressed and pyrexic. There was excessive salivation, difficulty in swallowing and anorexia. One-half of the experimental calves died from the infection. There were areas of focal necrosis throughout the alimentary tract. Eosinophilic inclusion bodies were found in nuclei of epithelial cells in and around the necrotic foci. Contents of the abomasum adhered to the mucosa.

McKercher (1963) made a comparative study of 3 European strains of Bläschenausschlag (coital vesicular exanthema) virus with an infectious bovine rhinotracheitis isolate recovered from an infected herd of dairy cattle in California. In cross-protection tests, the calves were immune to a challenge dose of heterologous virus. Sera from calves which were inoculated with each of 4 strains of virus cross reacted to the same extent with

infectious bovine rhinotracheitis virus in complement-fixation tests. The neutralizing indeces with all the viral isolates were very nearly the same. McKercher concluded that infectious bovine rhinotracheitis virus and Bläschenausschlag virus were the same.

The time and manner in which the virus entered United States remains unknown. It is almost certain that the virus was introduced from Europe prior to 1930, as that was the year an embargo was imposed on importation of cattle from continental Europe.

McKercher thought the reason that the virus was noted to cause only genital infection was because the animals in Europe were closely confined in small herds. Usually the only contact with animals outside of the herd was with the community bull. If the virus invaded the respiratory tract, it would not develop sufficient virulence in so few cattle to cause clinical respiratory disease. The fact that dairy herds tend to be maintained on a closed-herd basis may account for the disease being confined to the genital tract in dairy cattle in eastern United States.

Upon introduction into the western United States, the virus gained virulence because of the larger herd size and freedom of movement of cattle from herd to herd. Since most of the feedlot animals were either steers or reproductively inactive heifers, the disease was little noticed as infectious pustular

vulvovaginitis.

Studdert et al. (1964) became quite concerned over the fact that no one had reported infectious pustular vulvovaginitis in western United States. Since it was known that infectious pustular vulvovaginitis and infectious bovine rhinotracheitis occurred quite frequently, why had they not seen infectious pustular vulvovaginitis?

An outbreak of infectious pustular vulvovaginitis occurred in a sizeable dairy herd in California. Experimental heifers were inoculated with a viral isolate from this outbreak and a California strain of infectious bovine rhinotracheitis virus for comparison. In cross-protection tests those animals which were inoculated with one virus were resistant to a subsequent challenge dose of the other virus. Antiserum to each virus neutralized the other virus in cell cultures.

A great deal of mystery prevailed concerning the role of infectious bovine rhinotracheitis virus as the etiological factor in abortion. It was often suspected as being the cause but not fully confirmed. Crane et al. (1964) studied a herd of beef cattle in California in which there had been many abortions. A number of different causes of abortion were confirmed. On the basis of isolation and identification of infectious bovine rhinotracheitis virus from the aborted fetuses, they concluded that there was a prenatal infection initiated by infectious bovine rhinotracheitis virus. The dams which aborted

their fetus had serum titers which rose as high as 1:160.

McKercher and Wada (1964) expressed doubt that infectious bovine rhinotracheitis virus could cause abortion. Since the disease had been present in United States for 12 years without confirmed reports of abortion, it was thought that it could not cause abortion. Furthermore, the vaccine had been in use since 1957 without any known adverse affects. Nevertheless, many practitioners insisted that they had sufficient evidence that some abortions resulted from infectious bovine rhinotracheitis.

McKercher and Wada stated that a viremia was necessary for the virus to invade the uterus and so far many attempts to demonstrate viremia were unsuccessful. It is not known if their statement implies that some attempts to demonstrate viremia were successful. French (1962) isolated the virus from the blood stream. The virus, which is not highly concentrated in the blood, can be isolated quite readily from blood leucocytes. Even though French did not consider this state to be a true viremia, it was enough to dispel McKercher's disbelief. The virus could invade the uterus by adhering to circulating leucocytes. McKercher and Wada induced abortion in 6 out of 7 experimentally infected cattle. In conclusion, they said it was by inference that they believed the virus had undergone some change which enabled it to cause this new manifestation of disease but had not changed in its antigenic behavior.

An investigation by Chow et al. (1964) made it rather conclusive that infectious bovine rhinotracheitis virus could and did cause cows to abort their fetus. These investigators injected infectious bovine rhinotracheitis virus into pregnant heifers in various stages of pregnancy. The heifers became febrile and gave birth to still-born calves. The virus invaded the uterus, becoming well established before maternal antibody was formed. Since globulin does not cross the maternal-fetal barrier, the virus was uninhibited and caused abortion.

Apparently, Owen et al. (1964) did not agree with McKercher and Wada that infectious bovine rhinotracheitis virus had undergone some sort of change in order to cause abortion, but felt that this new disease-producing potential acquired by the virus had been present all the time and had been overlooked.

The work of Chow, Molello and Owen was continued by Owen, Chow and Molello. Previously, they studied the capability of infectious bovine rhinotracheitis virus to cause abortion and the susceptibility of cattle in the 1st and 3rd trimester of pregnancy. In continuance of their work, they studied the abortifacient effects of infectious bovine rhinotracheitis virus, the susceptibility of the fetus at different stages of development and the gross and microscopic changes in the fetus.

Heifers which had been inoculated responded with characteristic clinical signs of uncomplicated infectious bovine rhinotracheitis. Abortions occurred in the 1st and 3rd tri-

mester of pregnancy. Those fetuses which were aborted or dead *in utero* differed in appearance from those of normal heifers in that the skin was slightly edematous. Gross lesions could not be detected internally in the dead fetuses. Microscopic examination disclosed focal necrosis of hepatic and splenic cells. There was lymphocytic infiltration in the liver and spleen of aborted and dead fetuses in the 3rd trimester. Intranuclear inclusion bodies were not found in the spleen and liver. Respiratory and gastrointestinal mucosa were not examined for inclusion bodies.

As a result of their investigation, they recommended that the pregnant females not be vaccinated.

Madin *et al.* (1956) were the first to successfully isolate the virus of infectious bovine rhinotracheitis in bovine embryonic kidney cell culture from nasal exudate from experimentally infected calves which had been infected with nasal exudate from naturally infected beef and dairy cattle in southern California and from beef cattle in Colorado feedlots. The virus was cytopathogenic. They were unsuccessful in their attempts to isolate the virus in mice, guinea pigs and embryonated chicken eggs. They were able to reproduce an acute upper respiratory condition in cattle similar to that reported by Schroeder and Moys and Jensen *et al.* with the virus which was isolated from the nasal exudate. There was cross neutralization with the California agent and the Colorado isolate.

Freezer life of the virus was 7 months at -79°C. The virus remained viable for 96 hours at 37°C.

Persistence of the virus in infected tissues was studied by McKercher, Wada and Straub (1963). They isolated the virus from ocular tissue and from contiguous lymph nodes 6 days post-inoculation. The virus persisted in nasal secretions for as long as 9 days and in the larynx for 12 days. The virus was isolated from other tissues of the body but its presence was interpreted as resulting from circulating leucocytes.

Characterization of infectious bovine rhinotracheitis virus was advanced considerably as a result of the contribution by Armstrong, Pereira and Andrewes (1961). They suggest that infectious bovine rhinotracheitis virus be classified in the herpesvirus group because of the type of cytopathic effect it produces in cultured cells. Like other members of the herpes-virus group it is ether sensitive, a DNA virus and forms type A intranuclear inclusion in the cultured cells. The intranu-clear virus is enclosed by one membrane, and the cytoplasmic virus is enclosed by two membranes.

The growth rate, plaque formation, thermosensitivity, absorption kinetics and kinetics of viral replication were studied by Stevens and Groman (1963). They cultured the virus in Madin-Darby bovine kidney cell line (MDBK). According to their account, the virus appears in the cell 4 hours before burst time.

Later, Stevens and Groman (1964) noted that unabsorbed

virus was destroyed by trypsin. Plaques continued to enlarge in the presence of antiserum. Sonic treatment for 1 minute was as effective in causing the release of the virus from the cell as was freezing and thawing. Among their observations was the fact that intranuclear inclusions formed prior to the release of mature virus. Rounding of the infected cultured cell coincided with the formation and release of mature virus. Inclusions were noted as early as 8 hours postinoculation. The maximum virus titer was attained at 20 hours postinoculation in the MDBK cell line. Plaques were thought to be formed by: normal but unassayable infective virus that passes from cell to cell; subviral particles that pass between contiguous cells; a classical toxin which caused a self-sustaining molecular disorganization.

A statistically designed serological survey for infectious bovine rhinotracheitis antibodies in Massacheusetts cattle was conducted by Smith et al. (1964). They used random sampling to obtain the sample population. A stratified quota sample was used in which each county constituted a stratum. The number of serum samples from each county was in proportion to the number of cows within that county. About 100,000 cows were represented in the survey.

They found that 12.7 percent of all serum samples tested contained antibodies to infectious bovine rhinotracheitis virus and that 18.5 percent of all herds tested contained reactors. In most instances, a single positive serum sample accounted for

the reactor status of the herd. There were no reactors from vaccinated herds.

Kahrs et al. (1964) designed a serological survey of dairy cattle in New York. One thousand serum samples were allocated to 53 counties on the basis of their respective cattle populations. Since they did not receive the desired number of serum samples from each county, the contribution of each county to the state total had to be weighed to allow for missing serum samples. The average incidence per herd for infectious bovine rhinotracheitis was 13 percent.

Other reports of serological tests on collections of sera were made by Gillespie et al. (1957), Newberne et al. (1961), Greig (1961) and Niilo et al. (1962). The percentage of reactors ranges from 8.1 percent to 37 percent. It will be noted by these surveys that infectious bovine rhinotracheitis has become widespread and is often of subclinical nature.

MATERIALS AND METHODS

The Sample

The sample* was obtained from stock cow herds in Iowa between June 1, 1963, and May 31, 1965. About 85 percent of the sample was procured in the first 15 months. The sera were obtained from the State-Federal Brucellosis Laboratory during the period when the cattle population of Iowa was being tested under the Cooperative State-Federal Brucellosis Testing Program. The sample was limited to stock cows because this was the only class of cattle which would provide the most complete sample. That is, the researcher would have access to nearly the entire population of this class of cattle. Whether or not the cattle had brucellosis in no way had any bearing on this survey. It was very convenient to obtain the sera with the least amount of time and effort expended.

Feeder cattle would not provide a satisfactory sample as there would be so few specimens submitted from this group. Dairy cattle were excluded because the Brucellosis Ring Test was used quite generally to disclose reactor herds. Then, only sera from reactor herds or herds being dispersed by sale were submitted to the laboratory for testing. These factors virtually eliminated all but the stock cows. Even so, some were

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*Hereafter, sample, when used as such, refers to the entire collection of sera for this survey.

unavailable as they were calfhood vaccinated against brucellosis or that the animal had been tested quite recently and the grace period had not expired.

Further limitations to the sample size were encountered. Only a limited number of counties were made eligible to test. The original group consisted of 36 counties quite evenly distributed over the state. Two more were added shortly after the program got under way. This made a total of 38 (Fig. 1).

The size of the sample was based upon the Agriculture census of 1959. The census figures are tabulated in Table 1 of the appendix.

The sample was completely randomized. The test charts were examined daily and the number of serum samples received from each county was recorded separately. A running total was kept for each county so that the serum samples could be properly selected. Each of the 38 counties was given a 3-digit number called the "start number". The purpose of this was to avoid any bias which might be introduced by commencing the serum collection from each county on the same day at the same time.

For instance, the start number for Allamakee county was 599. It took 27 days to accumulate this total. The start number for Appanoose county was 453. This total was reached within 15 days after the first serum samples arrived.

A sample of 3 sera was taken from the herd that raised

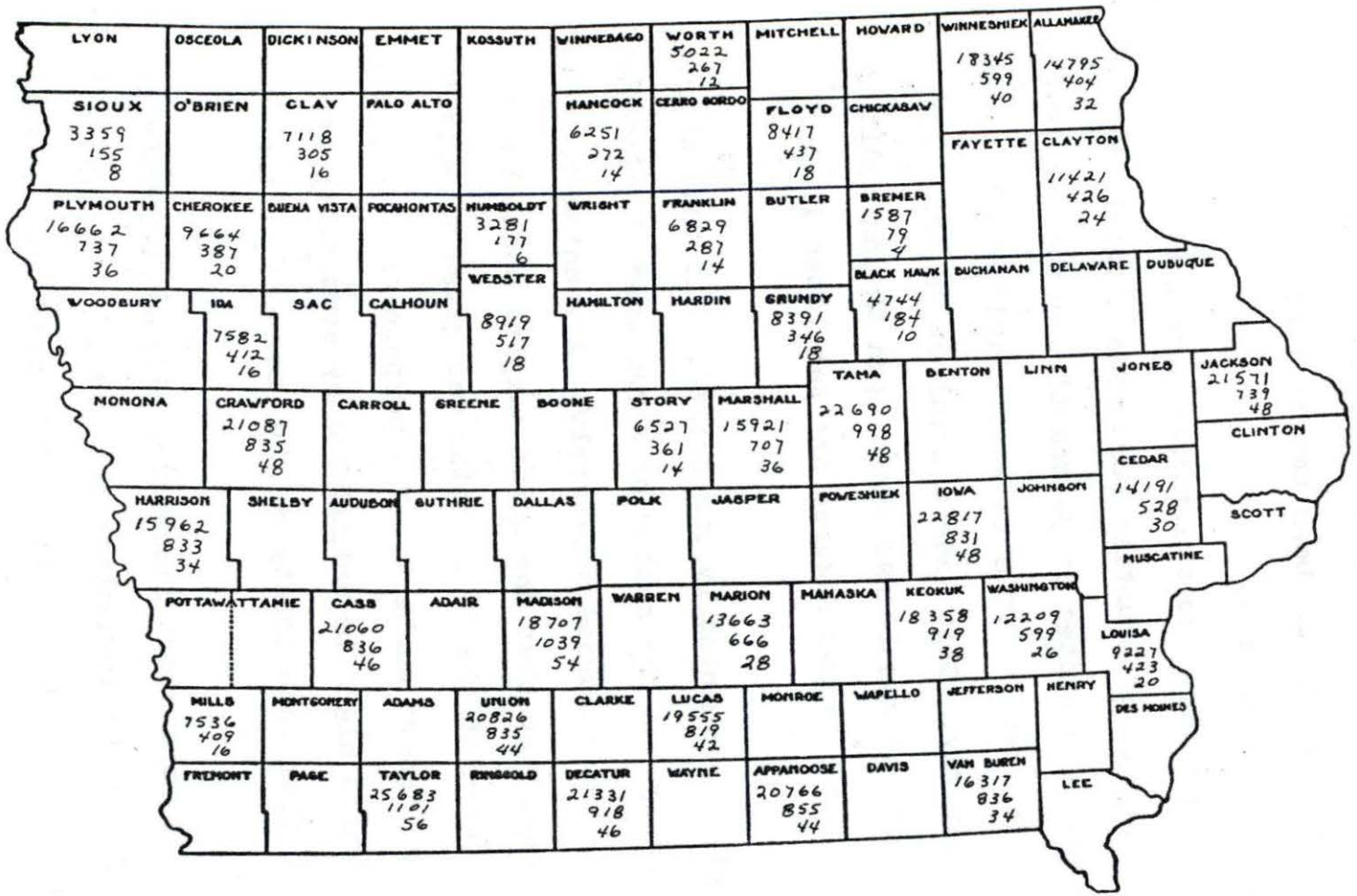

Figure 1. Counties from which the sample was taken. Top figure is the number of cows. Middle figure is the number of herds. Bottom figure is the number of serum samples.

the running total number of cows equal to, or greater than, the start number. From this point on each sample of 3 was taken at an interval of 700 until all of the serum samples from the county had been received. A maximum of 3 sera from each herd was included in the composite sample. This was determined by random selection. If there were only 3 cows, or less, in the herd all of the sera were included in the sample. Fortunately, all of the herds were at least 3 cows, or more, in size.

Each serum sample was coded with the number of the county from which the cattle originated and an accession number for that county. After separating the serum from the clot by centrifugation, the serum was heated for 30 minutes at 56°C. Several drops of penicillin-streptomycin solution were added to the serum according to the amount of serum saved. The tubes were stoppered, cartoned according to county, placed in the freezer and held at -20°C. until time of testing.

The sample resulted in a collection of 2205 sera. To test this many sera would be a task of much greater magnitude than could be accomplished in the alloted time. It was decided to decrease the sample by one-half. This was done by deleting 1 out of 4 herds and further deleting 1 cow from each of the 3 remaining herds. Four herds of 3 cows each made a total of 12 samples. Deletion of 1 herd left 9 samples. Deletion of 1 cow from each of the 3 remaining herds resulted in 6 samples for test. Thus, the sample was reduced by one-half.

The Virus

The Colorado strain of infectious bovine rhinotracheitis virus was obtained from Dr. C. E. Phillips.* A pool of this virus was grown on bovine testicle cell monolayers. It was stored in 1 ml. aliquots at -20°C. The titer of each batch was determined according to the method of Reed and Muench (1938) and appropriate dilution made so that 1000 50 percent tissue culture infective doses ($TCID_{50}$) were subjected to the neutralizing action of 1 ml. of test serum.

This particular batch of virus was prepared and frozen Oct. 31, 1965. The titer was 3.6×10^8. The virus was titered weekly commencing Jan. 6, 1966, (Table 2 of the appendix).

The potency of the virus was maintained for 90 days before it diminished noticeably. A change in potency of the virus was usually noted in the titration of the positive sera; the end-point was not as clearly defined. This weakness of the virus would have a tendency to create higher serum titers. Virus of low titer seemed to be equally as effective as virus of higher titer in determining positive or negative serum.

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The Cell Culture

Glassware

All glassware was soaked in Haemo-Sol* for at least 12 hours. Each item was brushed 10 to 12 times and rinsed 8 times in deionized distilled water. After the glassware had been dried in an oven at about 45°C., it was prepared for sterilization by autoclaving. The tops of the flasks were covered with aluminum foil. Test tubes were placed in racks and covered with paper tops fashioned for easy removal and reuse. The caps were placed open-end down in petri dishes. Syringes, forceps and scissors were wrapped in envelopes designed for autoclave sterilization.

Media

Hanks' and Earle's balanced salt solutions were used to establish and maintain the cell cultures. Each solution was compounded at 1X strength according to the following formulae:

Hanks' Balanced Salt Solution

NaCl	8	G.
KCl	0.4	G.
$CaCl_2$	0.14	G.
$MgSO_4 \cdot 7H_2O$	0.1	G.
$MgCl_2 \cdot 6H_2O$	0.1	G.

*Registered trademark of Meinecke and Co., Inc.

$Na_2HPO_4 \cdot 2H_2O$	0.06 G.
KH_2PO_4	0.06 G.
Glucose	1 G.
$NaHCO_3$	0.35 G.

Earle's Balanced Salt Solution

NaCl	6.8 G.
KCl	0.4 G.
$CaCl_2$	0.2 G.
$MgSO_4 \cdot 7H_2O$	0.2 G.
$NaH_2PO_4 \cdot H_2O$	0.125G.
Glucose	1 G.
$NaHCO_3$	2.2 G.

Lactalbumin hydrolysate was added to each formula at the rate of 5 grams per liter. Phenol red, 0.02 grams per liter, was added for pH indicator. All of the ingredients were weighed on an analytical balance and, with the exception of sodium bicarbonate, were combined in a volumetric flask and dissolved in deionized distilled water. Not all of the distilled water was added at this time. The flask was filled so that the water level was well below the neck. When the flask was filled too full it acted as a perculator and some of the contents would be lost in the autoclave. The flask was capped with a piece of aluminum foil and sterilized in the autoclave for 15 min. at 121°C. under 15 lbs. pressure. When

the contents of the flask had cooled sufficiently following sterilization sodium bicarbonate, which had been sterilized by filtration through a Selas 03 filter, was added and the entire volume extended to 1 liter by the addition of sterilized distilled water. Penicillin and streptomycin were added at the rate of 100 units and 100 mg. per ml. of medium, respectively.

Serum

One hundred milliliters of ovine serum were added to 900 ml. of balanced salt solution to provide growth medium for the cell cultures. The serum was processed from sheep blood from the market flock owned by Iowa State University. Several lambs were slaughtered at one time at the University's meat laboratory. The blood was collected in buckets and allowed to clot. The serum was poured off and centrifuged to remove cellular components and heavy impurities. The serum was passed through clarifying filters and sterilized by passing it through a D-8 Seitz filter. The sterilized product was bottled, frozen and held at -20°C. until it was thawed and incorporated in the balanced salt solution.

Trypsin solution

Two grams of trypsin 1:250* were combined in 1 liter of saline of Ham and Puck (1962):

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*Difco Laboratories, Detroit, Michigan

Glucose	1	G.
NaCl	8	G.
KCl	0.4	G.
$NaH_2PO_4 \cdot 7H_2O$	0.045	G.
KH_2PO_4	0.03	G.
$CaCl_2 \cdot 2H_2O$	0.016	G.

The solution was sterilized by filtration. It was bottled, frozen and stored at -20°C. until needed.

Calcium ions are very essential for the proper action of trypsin on bovine testicular tissue. The lack of calcium ions results in the formation of a soft gel-like mass instead of a uniform suspension of cells.

Cell source

Bovine testicles served as the source of cells for the cell cultures. The testicles were obtained at the abattoir from veal calves at the time of slaughter. The testicle was removed from the carcass with both the tunica communis and tunica vaginalis intact with 3 to 4 inches of spermatic cord attached. By leaving the tunics intact and a stump of spermatic cord, the natural sterile environment within these structures was preserved.

Upon removal of the tissues, they were placed in plastic bags, iced and delivered to the laboratory during the next 12 to 16 hours. This time in transit did not seem to have any appreciable affect on the viability of the cell.

Cell culture procedure

It is a tedious process to remove the parenchymatous tissue from within the testicle without contaminating it. The operator must be meticulous. With practice one can become quite proficient at this task.

Instruments required to obtain the parenchymatous tissue from the testicle are sterile petri dish, scalpel, 2 curved thumb forceps, scissors, hemostat, 100 ml. beaker filled with 70 percent ethyl alcohol and bunsen burner. The alcohol will serve to sterilize the instruments during the process of removing the parenchyma. Instruments are left in alcohol until needed. The alcohol is removed by touching the instrument to the flame and allowing the alcohol to burn off. Repetition of this flaming process 3 times is sufficient to heat sterilize the instruments and also remove all of the alcohol. The temper and finish of the instruments are not damaged by this method.

Excess fat, hair and fascia are removed from the testicle. Assuming one has more control over the right hand than the left, grasp the hemostat with the left hand. The testicle is positioned so that the tunic attachments running along the long axis of the testicle are toward the operator. The ligamental structures are grasped with a thumb forceps in the right hand. The forceps are transferred to the left hand and positioned between the thumb and index finger. The hemostat is held in the palm of the left hand extending between the last

2 fingers. If properly held, one can spread the fingers and produce tension on the cord.

The cord is severed in one bold cut at the epididymis with scissors which have been flamed. This must be done so as not to expose any of the parenchymatous tissue. The naturally sterile field must be preserved. The tension causes the tunica vaginalis to draw back upon the testicle and expose the tunica communis. The outer tunic is completely retracted with the fingers taking care not to touch the tunica communis. The tunica vaginalis can be removed at its ligamental attachment by manipulating the hemostat and thumb forceps.

The hemostat is repositioned at the same location on the testicle taking care to put tension on the tunica communis so that when it is cut the internal contents are forced outward. The tunica communis is cut with scissors from the same end as before but close enough to expose the parenchymatous substance. By applying pressure with the fingers the contents can be squeezed out. One must be cautious and exercise care so as not to contaminate the exposed tissue. The contents are removed with a sterile forceps that have not been previously used and transferred to the petri dish.

There is a central core of dense connective tissue within each internal mass. It is well to remove this as it will not yield many cells and serves only to bind a certain amount of proteolytic activity of the trypsin. This can be done by

teasing the soft tissue from the core of dense tissue.

The teased tissue and trypsin are combined in a trypsinizing flask placed on a magnetic stirrer for masceration which takes about 15 minutes. The coarse shreds of tissue are filtered out on sterile gauze. The filtrate is centrifuged at 1000 rpm. for 10 minutes. The supernatant is decanted. The sedimented cellular portion is suspended in Hanks' balanced salt solution. A hemocytometer is charged with the cell suspension and the number of cells per milliliter is determined. The cell suspension is standardized at 3,000,000 per ml. in Hanks' balanced salt solution containing 0.5 percent lactalbumin hydrolysate, phenol red and 10 percent ovine serum. One milliliter aliquots are placed in screw-cap roller tubes which were sterilized in test tube racks with paper covers adapted for this purpose.

The entire rack of tubes is filled at one time with a self-filling pipette. Each tube is capped and placed in a 72-space rack for incubation. In order to maintain the tubes in an oblique position, it is necessary to place a cardboard spacer between each row of tubes. This gives the same degree of slant to each row of tubes. The seeded tubes are incubated at 37°C. in a conventional incubator. Since the tubes are sealed with rubber-lined screw caps, no special gas environment is required.

The medium is literally poured off and replaced after the first 24-hour incubation period. A confluent monolayer devel-

opes within 72 hours. The monolayer is suitable for inoculation at this stage.

Serum Neutralization

Four-tenths milliliter of test serum was delivered into a clean test tube. An equal amount of infectious bovine rhinotracheitis virus which had been diluted with Hanks' balanced salt solution to contain 2000 $TCID_{50}$ per ml. was added to the serum. This constituted the 1:2 dilution of the serum. Each 0.1 ml. of the resultant mixture which became the inoculum for the monolayer contained 100 $TCID_{50}$. The test serum and virus were allowed to react at room temperature for a period of 30 minutes.

Inoculation of the Cell Monolayer

Each of 5 tubes containing a confluent monolayer was inoculated with 0.1 ml. of the inoculum prepared in the serum neutralization step. The tube was appropriately labeled and incubated in an oblique position for 30 minutes at 37°C. to permit absorption of unneutralized virus by the cells of the monolayer. One milliliter of Earle's balanced salt solution containing phenol red and 0.5 percent lactalbumin hydrolysate was added to the test tubes following the absorption period. Again the tubes were returned to the incubator and incubated in a stationary oblique position at 37°C. for 72 hours

The Cytopathic Effect

Infectious bovine rhinotracheitis virus causes a cytopathic effect in the monolayer. The affected cells become more refractile, round instead of spindle-shaped and loosen from the glass. This phenomenon may occur in small isolated areas or become quite diffuse throughout the entire monolayer. Sometimes the entire monolayer is loosened and slides down the tube in a film.

Prior to the actual testing of the suspect serum, preliminary trials were made with known positive and negative serum to determine the number of $TCID_{50}$ which would give the best results. Actually, there was no significant difference in the results with 50 $TCID_{50}$ to 200 $TCID_{50}$. For ease of calculation it was decided that the infective dose of virus would be 100 $TCID_{50}$.

Following the 72-hour incubation period, each tube which had been inoculated with the serum-virus mixture was examined under the microscope at 100X. Presence of the cytopathic effect in the cell monolayer was used as the basis for classifying the animal from which the test serum originated a non-reactor. This is to mean that the animal did not have serum neutralizing antibodies for infectious bovine rhinotracheitis virus. Serum containing neutralizing antibodies rendered the virus inactive, permitting the inoculated cell monolayer to develope normally. Donors of this serum were considered to be

reactors.

Control cultures, uninoculated and inoculated with virus only, were included with each group of tests.

Serum Titer

All sera were assayed at 1:2 dilution. Those which were found to contain antibodies to infectious bovine rhinotracheitis virus were further examined to determine the concentration of antibodies present. Each serum reacting at the 1:2 dilution was further diluted commencing with 1:4 and continuing through 1:64, or greater, if necessary. Routinely, the serum was tested at 1:4, 1:8, 1:16, 1:32, and 1:64 dilutions by the procedure described above.

RESULTS

From the serum-neutralization tests assayed in bovine testicle cell culture it was found that 24.1 percent (Fig. 2) of the 1106 samples tested contained antibodies to infectious bovine rhinotracheitis virus and that 36.3 percent (Fig. 3) of the herds tested were reactor herds. In 33.3 percent of the reactor herds both cows representing the herd reacted positively; whereas in 66.2 percent of the reactor herds only 1 of the 2 cows was positive. There were no reactor animals in 63.8 percent of the herds tested. The greatest number of reactors occurred at the 1:2 dilution. Forty-four and one-half percent of the reacting cows reacted at this dilution. In subsequent order 18.7 percent reacted at 1:4; 17.6 percent at 1:8; 10.8 percent, at 1:16; 7.8 percent, at 1:32; none, at 1:64; and 0.37 percent, at 1:128. These results are recorded in Table 5 of the appendix. The highest titer, 1:128, occurred in a cow from Allamakee county.

Occasionally a serum was found to contain an overwhelming population of bacteria which had not been destroyed by pasteurization of the serum and bacteriocidal action of antibiotics. This difficulty was overcome by rerunning the test and reading the results at 24-, 36-, 48- and 60-hour intervals. The cytopathic effect in a negative serum usually was detectable at 36 hours after inoculation. Sometimes the serum was tentatively classified as positive and a titration was made. Dilution of

the serum resulted in diluting the microorganisms, and as a result the cytopathic effect could be more clearly detected at higher dilutions.

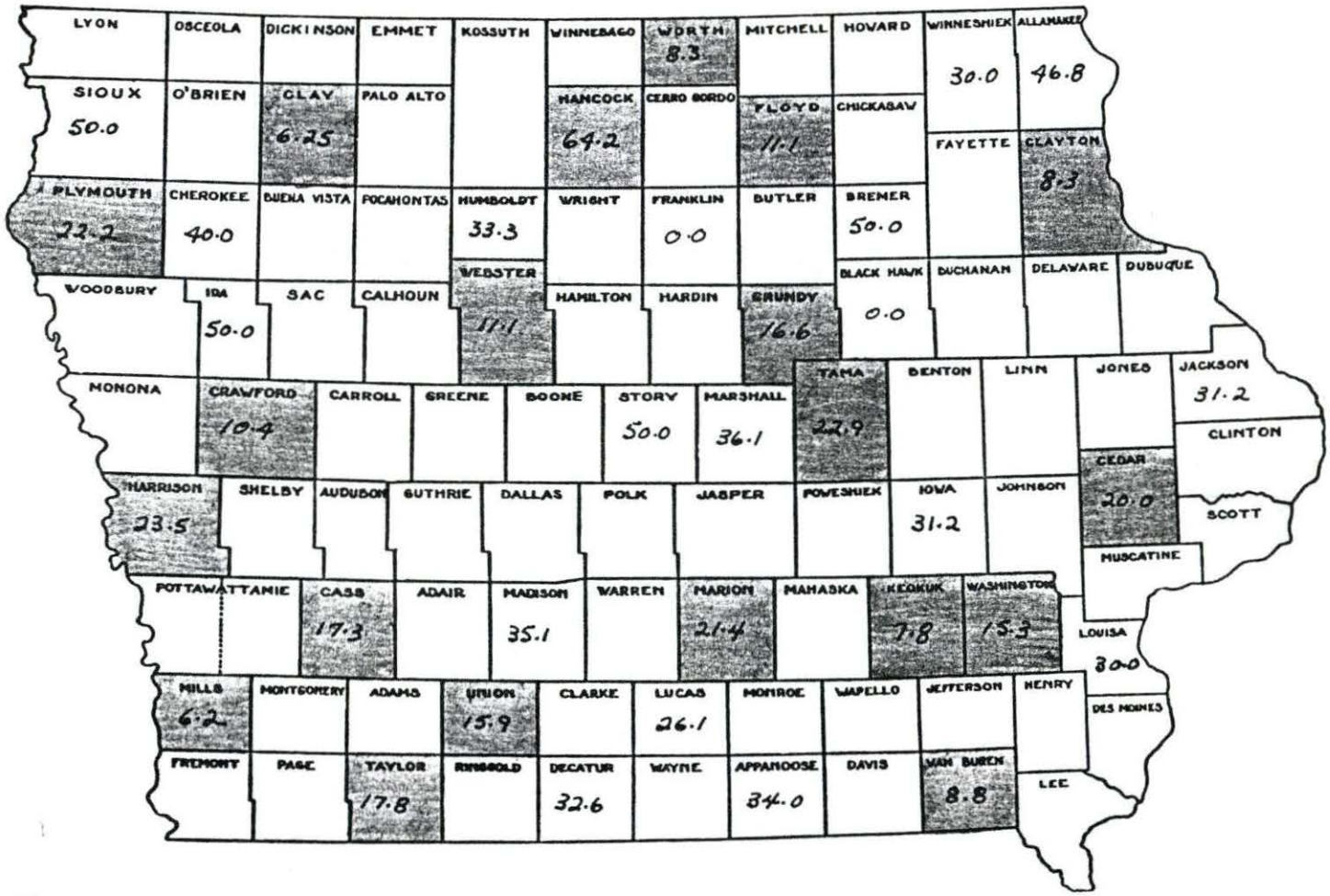

Figure 2. Per cent infected cows

1 to 25%
26 to 50%
51 to 75%
above 75%

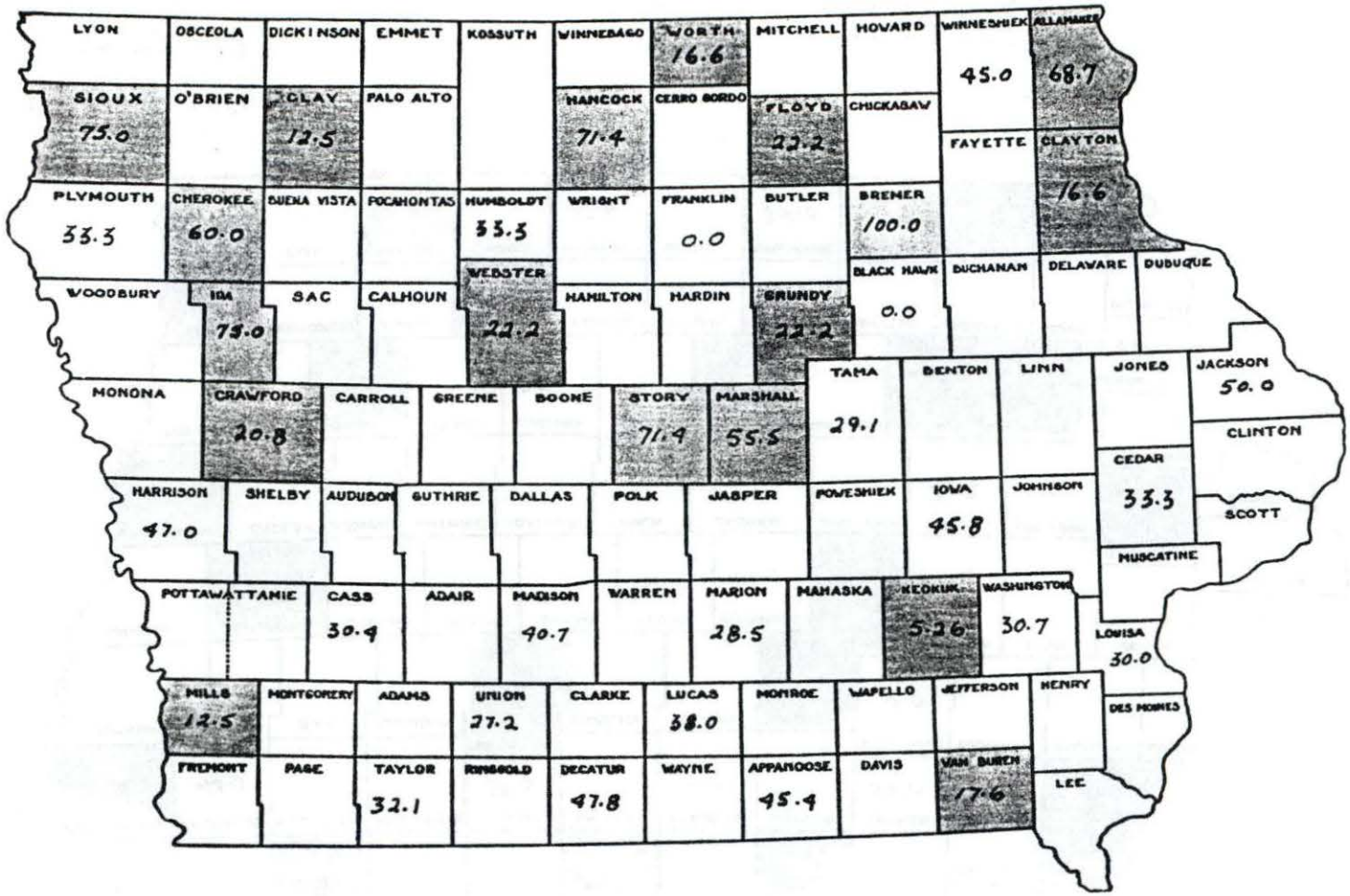

Figure 3. Per cent of sample pairs infected

1 to 25%
26 to 50%
51 to 75%
above 75%

DISCUSSION

This study was undertaken to ascertain the incidence of infectious bovine rhinotracheitis in Iowa stock cows by serum-neutralization tests in cultured bovine testicle cells. Random sampling was used enabling one to obtain reasonably accurate results by testing a relatively few serum samples. There are 514,919 cattle from 22,078 herds represented by the sample. From the serum-neutralization tests it was found that 24.1 percent of the 1106 sera tested contained antibodies to infectious bovine rhinotracheitis virus and that 36.3 percent of the herds represented contained reactor animals.

The statistical design of this survey places it in a category comparable to the work of Smith et al. (1964) and Kahrs et al. (1964). Smith et al. took their sample in Massachusetts at random from a stratified quota in which each county constituted a stratum. The sample, taken from 10 percent of the cows from 10 percent of the herds in a county, represented about 100,000 cows. They found that 12.7 percent of all serum samples tested contained antibodies to infectious bovine rhinotracheitis virus and that 18.5 percent of all herds tested contained reactors. Kahrs et al. designed a survey of dairy cattle in New York in which 1000 serum samples were allocated to 53 counties on the basis of their respective cattle populations. Their survey was somewhat weakened because they were unable to acquire all of the allotted serum samples. They

found the average incidence per herd for infectious bovine rhinotracheitis was 13 percent.

For practical purposes the rate of infection as found by Smith and Kahrs, 12.7 percent and 13 percent, respectively, is the same. The rate of infection, 24.1 percent, in the Iowa cattle as determined in this survey is nearly twice as great as that in Massachusetts and New York cattle.

Several more limited surveys for antibodies to infectious bovine rhinotracheitis virus have been reported. These were either restricted to a small area or to only a few herds. Gillespie et al. (1957) surveyed 43 herds of New York dairy cattle and found 12 percent of the herds had cows with neutralizing antibodies to infectious bovine rhinotracheitis virus. A similar survey of 53 herds was made for neutralizing antibodies to infectious pustular vulvovaginitis virus in which 15 percent of the herds were found to contain reactor animals. Greig (1961) obtained a random collection of bovine sera from cattle in southern Ontario, Canada. He tested 1365 sera and found 8.1 percent of the cows and 18.9 percent of the herds had antibodies to infectious bovine rhinotracheitis virus. Niilo et al. (1962) tested 1000 bovine sera from 55 herds representing 50 percent of the cattle in Alberta, Canada. He found neutralizing antibodies in 37 percent of the cows. Newberne et al. (1961) tested 2190 sera of which 35 percent contained antibodies to infectious bovine rhinotracheitis virus. These sera had been submitted to the laboratory for various diagnostic

tests. Many of the sera were from clinically affected animals.

The incidence of infection ranges from 8.1 percent to 37 percent depending upon the location of the cattle and the nature of the serum samples.

What factors are present (or absent) in Iowa that differ from Massachusetts and New York that cause or contribute to this greater incidence of infectious bovine rhinotracheitis? McKercher thought small herds and fewer replacements within the herd kept the virus at low virulence and reduced the spread of the disease. This statement would apply to the eastern cattle herds. It could very well apply to the Iowa stock cow herds. Replacements are usually off-spring of cows within the herd. Occasionally a few new heifers are introduced into the herd, and the herd sire is replaced periodically.

Could it be that the large feeder cattle population which is imported into Iowa through salesbarns and central markets is a source of infectious bovine rhinotracheitis? Many Iowa stock cow owners purchase feeder cattle to be fed along with their locally raised calves and yearlings. Even though these new cattle do not often mingle with the breeding animals, they are present on the premises and may share the watering tank with the breeding herd. The breeding herd being in such close proximity to the feeder animals could contact infectious bovine rhinotracheitis. Since cattle have a tendency to muzzle each other, this could place a susceptible animal in direct contact with infective material. Virus which is eliminated in nasal

exudate from infected individuals may be discharged into the watering tank, and in this way susceptible cattle would be indirectly exposed to infectious bovine rhinotracheitis virus.

It seems as though the disease is more contagious and more easily transmitted in the respiratory form than in the genital form. The respiratory form had not been recognized in eastern United States until 1959, although workers in eastern United States and Europe had been well acquainted with the genital form for several years. The lower incidence of infectious bovine rhinotracheitis and/or infectious pustular vulvovaginitis in eastern cattle is indicative of the less contagious genital form. The respiratory form was first diagnosed in Colorado feeder cattle and then in California dairy cattle. Cattle operations in both of these areas are on a large scale. There is continuous movement of cattle in and out of the herd. Such traffic is lacking in the eastern cattle herds. The way in which feeder cattle are moved about, intermingled and housed is quite conducive to the spread of infectious bovine rhinotracheitis, and one could expect to find a higher incidence of the respiratory form of infectious bovine rhinotracheitis in the feeder cattle of the West and Midwest, particularly Iowa.

If one were to draw an imaginary line in an east-west direction about midway through Iowa, dividing the state into a northern section and a southern section, all of the counties with 51 percent, or more, herd infection are found in the

northern section. Many more feeder cattle are shipped into the northern section of the state than the southern section. The scope of the present investigation does not allow more than speculation on this point. Additional research would be necessary to establish the relationship, if any, of feeder cattle traffic to the incidence of infectious bovine rhinotracheitis.

Smith et al. obtained herd history by way of a questionnaire and concluded that the cattle in their survey were clinically healthy. There was no history readily available for any of the herds in the Iowa survey or for the herds in the survey by Kahrs et al. These, too, were assumed to be clinically healthy cattle. The initial and primary purpose for obtaining these sera was for the brucellosis test and not because the animals were suspected of having infectious bovine rhinotracheitis. However, one must keep in mind that some individuals may have been convalescing from a recent infection of infectious bovine rhinotracheitis.

Once serological data has been acquired, they require an interpretation. What does it mean to find circulating neutralizing antibody to infectious bovine rhinotracheitis virus in the serum? How did the animal acquire these antibodies? Was it as the result of an active infection? Or was it due to vaccination reaction?

The levels of detectable antibodies, which are considered to be significant, vary in different diagnostic tests. Neutralization titers of 1:2, or higher, in the serum of cattle are

associated with infection with infectious bovine rhinotracheitis virus. Such infection may be the result of either natural exposure or vaccination with a modified live virus.

Although it may not have been general practice to vaccinate Iowa stock cows, it is quite possible that some cows may have been recently vaccinated against infectious bovine rhinotracheitis. What allowances should be made for the presence of antibodies to infectious bovine rhinotracheitis virus resulting from vaccination? According to the literature cited, there is general agreement that there is no serum titer following the administration of a single dose of vaccine under field conditions. However, Fastier and Smith (1962) demonstrated serum titers ranging from 1:8 to 1:32 30 days following vaccination.

Fastier and Smith administered the vaccine intramuscularly in preference to intranasal instillation. This method of inoculation resulted in establishing a state of immunity without causing respiratory symptoms and without disseminating the virus in the nasal exudate. In order to abolish the property of causing respiratory symptoms following intranasal instillation the virus would have to be so greatly attenuated that its immunogenicity would be destroyed. One should keep in mind that Fastier and Smith were working with a New Zealand strain of infectious bovine rhinotracheitis virus and in an environment unlike that of the United States. Perhaps the New Zealand strain of virus does engender detectable antibodies following

Serum neutralization tests to detect the presence of circulating antibody to infectious bovine rhinotracheitis virus have been performed at the Iowa Veterinary Diagnostic Laboratory for 18 months.* There have been 507 tests made of which 27.6 percent were positive. Most of the donors of the serum were not vaccinated or the date of vaccination was not recent enough for the owner to recall that the cattle had been vaccinated. In one specific instance a herd of feeder cattle had been injected with infectious bovine rhinotracheitis vaccine on Nov. 11, 1965, and again on Jan. 1, 1966, with a combination of infectious bovine rhinotracheitis and bovine virus diarrhea vaccine. Serum was obtained on January 10 from 1 animal. Serum from this animal did not contain neutralizing antibody for infectious bovine rhinotracheitis virus. The serum did have an antibody titer of 1:64 to bovine virus diarrhea virus. The cattle were clinically sick at the time and were thought to have virus diarrhea. In a second case, the herd had been injected with infectious bovine rhinotracheitis vaccine 30 days prior to obtaining serum. No neutralizing antibodies to infectious bovine rhinotracheitis virus were found in this sera. Many other examples could be cited. There is usually no detectable neutralizing antibody in the serum

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*Firkins, G. S. Iowa Veterinary Diagnostic Laboratory. Ames, Iowa. Serological studies on bovine abortion. Unpublished data. 1966.

following vaccination. Therefore, any neutralizing antibody which can be detected is an indication that the host has undergone either a clinical or inapparent infection due to infectious bovine rhinotracheitis virus.

Knowing that the consequence of vaccinating pregnant cows to protect them from contracting infectious bovine rhinotracheitis is to create an abortion storm in the herd, it is quite unlikely that the donors of the sera from this survey were immunized against infectious bovine rhinotracheitis virus. Therefore, it is concluded that the serum titers were induced by an active infectious process which in most cases was inapparent and went unnoticed.

Again, one's attention is directed to the work of Fastier and Smith. They state that abortion caused by infectious bovine rhinotracheitis virus is more of an inference rather than an established fact. In support of their stand they cited the work of Kendrick et al. (1958). Perhaps they were justified in making this statement, as most of the proof which established infectious bovine rhinotracheitis virus as a cause of bovine abortions had as yet to make its appearance. Certainly the work of Baker, McEntee and Gillespie (1960), Crane et al. (1964), Chow, Molello and Owen (1964) and Owen, Chow and Molello (1964) proved beyond any doubt that infectious bovine rhinotracheitis virus was responsible for bovine abortions.

At the time of this writing there are several herds of dairy cows and one herd of stock cows under investigation by the Iowa Veterinary Diagnostic Laboratory in an attempt to determine the cause of several abortions in the herd.* Most of the time the practicing veterinarian is unable to find the cause. The cows have not exhibited any outward signs of sickness until they abort their fetus. The sera have been negative to the Brucellosis and leptospirosis tests. No bacterial pathogens have been isolated from the fetuses. Nearly all of the sera contain antibodies to infectious bovine rhinotracheitis virus on initial tests. In one instance the antibody titer increased from 1:4 on initial test to 1:32 three weeks later, an 8-fold increase. Other sera have antibody titers from 1:2 to 1:256.

These case reports are not a part of the survey but are offered as evidence that infectious bovine rhinotracheitis virus is a very real etiological agent of abortion which may surpass the ability of all other abortifacients heretofore discovered. Furthermore, it seems quite possible that one can make a substantial diagnosis incriminating infectious bovine rhinotracheitis virus as the cause of abortion on the basis of serological tests.

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*Firkins, G. S. Iowa Veterinary Diagnostic Laboratory. Ames, Iowa. Serological studies on bovine abortion. Unpublished data. 1966

The incidence of infectious bovine rhinotracheitis, 24.1 percent, as revealed by this survey is an alarming rate of infection. Consider for the moment that at the beginning of State-Federal eradication in 1934, the incidence of bovine brucellosis in Iowa and throughout the United States was estimated at 10 percent (Lash and O'Rear, 1942). The extent of infection of bovine tuberculosis, a much less contagious disease, was only 4.2 percent in 1922, at its peak of infection (Mohler, 1942).

There were no specific factors provided by this survey which may account for this high rate of infection. Postvaccination titer could not be demonstrated. It is assumed that the serum donors were clinically healthy cattle, some of which may have been convalescing from a recent infection of infectious bovine rhinotracheitis. No assignment of factors responsible for infectious bovine rhinotracheitis could be made on the basis of location as the rate of infection at the county level was very erratic.

CONCLUSION

It has been revealed by this survey that 24.1 percent of the sera tested contained antibodies to infectious bovine rhinotracheitis virus. Due to the inability to demonstrate post-vaccination titer to infectious bovine rhinotracheitis virus, the figure presented, 24.1 percent, represents essentially the results of natural exposure to the virus. The high serological incidence is compatible with the hypothesis that this virus exists in an inapparent form. Antibodies to infectious bovine rhinotracheitis virus were found in cattle throughout the state. Except for the general grouping of high incidence counties in the northern section of the state, no pattern of infection could be related to geographical area or type of farming.

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APPENDIX

Table 1. A list of the counties with the number of herds and cows as tabulated from the 1959 Agriculture Census and the actual number of herds and cows recorded in the sample

County	Number of herds		Number of cows	
	Expected	Actual	Expected	Actual
Allamakee	224	404	11509	14795
Appanoose	301	855	14266	20766
Black Hawk	190	184	5224	4744
Bremer	108	79	2023	1587
Cass	342	836	15532	21060
Cedar	302	528	11982	14191
Cherokee	210	387	5698	9664
Clay	280	305	6451	7118
Clayton	279	426	9250	11421
Crawford	302	835	13625	21087
Decatur	256	918	14950	21331
Floyd	258	437	5512	8417
Franklin	286	287	6957	6829
Grundy	252	346	8863	8391
Hancock	313	272	6664	6251
Harrison	348	833	7501	15962
Humboldt	190	177	2623	3281
Ida	279	412	9250	7582
Iowa	391	831	18632	22817
Jackson	406	739	17867	21571
Keokuk	416	919	14058	18358
Louisa	196	423	6585	9227
Lucas	321	819	12938	19555
Madison	504	1039	18707	25235
Marion	352	666	11247	13663
Marshall	457	707	13110	15921

Table 1. (Continued)

County	Number of herds Expected	Number of herds Actual	Number of cows Expected	Number of cows Actual
Mills	179	409	5431	7536
Plymouth	394	737	11457	16662
Sioux	132	155	3704	3359
Story	325	361	5894	6527
Tama	505	998	18604	22690
Taylor	346	1101	17783	25683
Union	281	835	15475	20826
Van Buren	323	836	11757	16317
Washington	276	599	9123	12209
Webster	515	517	8972	8919
Winneshiek	356	599	11063	18345
Worth	249	267	5644	5022
Total	11644	22078	395931	514919

Table 2. Titer of virus expressed in $TCID_{50}$/1 ml.

Time	Decimal dilution of virus -1	-2	-3	-4	-5	-6	-7	-8	-9	Titer $TCID_{50}$/1 ml.
Initial	5/5	5/5	5/5	5/5	5/5	5/5	4/5	1/5	0/5	3.2×10^8
60 days	5/5	5/5	5/5	5/5	5/5	5/5	2/5	1/5	0/5	1.0×10^8
70 days	5/5	5/5	5/5	5/5	5/5	5/5	1/5	0/5	0/5	4.1×10^7
77 days	5/5	5/5	5/5	5/5	5/5	4/5	2/5	0/5	0/5	4.8×10^7
95 days	5/5	5/5	5/5	5/5	5/5	0/5	0/5	0/5	0/5	3.2×10^6
102 days[a]	5/5	5/5	5/5	3/5	2/5	0/5	0/5	0/5	0/5	3.2×10^5
109 days	5/5	5/5	5/5	5/5	5/5	1/5	0/5	0/5	0/5	4.1×10^6

[a]Cell sheet one day older than usual

Table 3. Size of sample and proportion of infected cows by counties

County	Total	Reactors	Negative	% Infected
Allamakee	32	15	17	46.87
Appanoose	44	15	29	34.09
Black Hawk	10	0	10	00.00
Bremer	4	2	2	50.00
Cass	46	8	38	17.39
Cedar	30	6	24	20.00
Cherokee	20	8	12	40.00
Clay	16	1	15	6.25
Clayton	24	2	22	8.33
Crawford	48	5	43	10.41
Decatur	46	15	31	32.60
Floyd	18	2	16	11.11
Franklin	14	0	14	00.00
Grundy	18	3	15	16.66
Hancock	14	9	5	64.28
Harrison	34	8	26	23.52
Humboldt	6	2	4	33.33
Ida	16	8	8	50.00
Iowa	48	15	33	31.25
Jackson	48	15	33	31.25
Keokuk	38	3	35	7.89
Louisa	20	6	14	30.00
Lucas	42	11	31	26.19
Madison	54	19	35	35.18
Marion	28	6	22	21.42
Marshall	36	13	23	36.11
Mills	16	1	15	6.25
Plymouth	36	8	28	22.22

Table 3. (Continued)

County	Total	Reactors	Negative	% Infected
Sioux	8	4	4	50.00
Story	14	7	7	50.00
Tama	48	11	37	22.91
Taylor	56	10	46	17.85
Union	44	7	37	15.90
Van Buren	34	3	31	8.82
Washington	26	4	22	15.38
Webster	18	2	16	11.11
Winneshiek	40	12	28	30.00
Worth	12	1	11	8.33
Total	1106	267	839	24.14

Table 4. Number of sample pairs tested and proportion infected by counties

County	Total	Reactor	Negative	% Infected
Allamakee	16	11	5	68.75
Appanoose	22	10	12	45.45
Black Hawk	5	0	5	00.00
Bremer	2	2	0	100.00
Cass	23	7	16	30.43
Cedar	15	5	10	33.33
Cherokee	10	6	4	60.00
Clay	8	1	7	12.50
Clayton	12	2	10	16.66
Crawford	24	5	19	20.83
Decatur	23	11	12	47.82
Floyd	9	2	7	22.22
Franklin	7	0	7	00.00
Grundy	9	2	7	22.22
Hancock	7	5	2	71.42
Harrison	17	8	9	47.05
Humboldt	3	1	2	33.33
Ida	8	6	2	75.00
Iowa	24	11	13	45.83
Jackson	24	12	12	50.00
Keokuk	19	2	17	5.26
Louisa	10	3	7	30.00
Lucas	21	8	13	38.09
Madison	27	11	16	40.74
Marion	14	4	10	28.57
Marshall	18	10	8	55.55
Mills	8	1	7	12.50
Plymouth	18	6	12	33.33

Table 4. (Continued)

County	Total	Reactor	Negative	% Infected
Sioux	4	3	1	75.00
Story	7	5	2	71.42
Tama	24	7	17	29.16
Taylor	28	9	19	32.14
Union	22	6	16	27.27
Van Buren	17	3	14	17.64
Washington	13	4	9	30.77
Webster	9	2	7	22.22
Winneshiek	20	9	11	45.00
Worth	6	1	5	16.66
Total	553	201	352	36.34

Table 5. The distribution of serum titers of reactor cows by counties

County	Total tests	Number reactors	Serum dilution 1:2	1:4	1:8	1:16	1:32	1:64	1:128
Allamakee	32	15	4	1	3	3	3	0	1
Appanoose	44	15	8	1	2	1	3	0	0
Black Hawk	10	0	0	0	0	0	0	0	0
Bremer	4	2	1	0	0	1	0	0	0
Cass	46	8	3	2	1	2	0	0	0
Cedar	30	6	3	0	3	0	0	0	0
Cherokee	20	8	4	1	1	0	2	0	0
Clay	16	1	0	1	0	0	0	0	0
Clayton	24	2	1	1	0	0	0	0	0
Crawford	48	5	1	3	0	0	1	0	0
Decatur	46	15	10	1	2	2	0	0	0
Floyd	18	2	1	1	0	0	0	0	0
Franklin	14	0	0	0	0	0	0	0	0
Grundy	18	3	0	0	3	0	0	0	0
Hancock	14	9	4	2	1	1	1	0	0
Harrison	34	8	8	0	0	0	0	0	0
Humboldt	6	2	2	0	0	0	0	0	0
Ida	16	8	7	0	0	0	1	0	0
Iowa	48	15	5	4	2	1	3	0	0
Jackson	48	15	7	2	4	2	0	0	0

Table 5. (Continued)

County	Total tests	Number reactors	Serum dilution 1:2	1:4	1:8	1:16	1:32	1:64	1:128
Keokuk	38	3	2	0	0	0	1	0	0
Louisa	20	6	3	1	2	0	0	0	0
Lucas	42	11	5	3	3	0	0	0	0
Madison	54	19	10	5	1	2	1	0	0
Marion	28	6	4	2	0	0	0	0	0
Marshall	36	13	3	6	4	0	0	0	0
Mills	16	1	0	0	1	0	0	0	0
Plymouth	36	8	1	1	6	0	0	0	0
Sioux	8	4	2	1	0	1	0	0	0
Story	14	7	2	0	0	3	2	0	0
Tama	48	11	4	1	2	4	0	0	0
Taylor	56	10	4	1	1	3	1	0	0
Union	44	7	5	1	1	0	0	0	0
Van Buren	34	3	2	1	0	0	0	0	0
Washington	26	4	1	1	1	1	0	0	0
Webster	18	2	1	1	0	0	0	0	0
Winneshiek	40	12	1	4	3	2	2	0	0
Worth	12	1	0	1	0	0	0	0	0
Total	1106	267	119	50	47	29	21	0	1
% Total		24.1	44.5	18.7	17.6	10.8	7.8	0.0	0.37